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Development of methods using phytoalexin (resveratrol) assessment as a selection criterion to screen grapevine *in vitro* cultures for resistance to grey mould (*Botrytis cinerea*)

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Summary

The purpose of this research was, firstly to determine the ability of grapevine *in vitro* cultures to synthesize resveratrol, a stilbene-type phytoalexin that is considered to be a good marker for resistance of grapevines to *Botrytis cinerea*, the causal organism for grey mould. Secondly, this study sought to establish the relationship between phytoalexin production potential and resistance to *Botrytis cinerea* in grapevines. In this aim, resveratrol production was assessed in 13 *Vitis* species or cultivars. A good correlation appeared between resveratrol production by grapevine *in vitro* cultures and grey mould resistance except for two *Vitis* spp. for which no correlation was observed, thus suggesting that resistance of grapevines may sometimes be associated with factors other than stilbene phytoalexins. In view of the results obtained, the potential use of resveratrol induction and of *in vitro* methods as a tool for screening grapevines for resistance to *B. cinerea* was discussed.

Introduction

Grey mould (*Botrytis cinerea* Pers: Fr) is an important disease causing serious losses to a wide range of plants. In vineyards, particularly, the disease affects both the yield of grape berries and the quality of wine.

Techniques available for breeding for grape disease resistance in the case of the resistance to *B. cinerea* may include intraspecific hybridization, use of somaclonal variation and gene insertion (Mullins et al., 1992).

Conventional genetic improvement has proved to be of limited use as the vine requires broad heterozygosity (Bessis, 1986). When this is lessened by self-fertilization, inbreeding rapidly leads to loss of the plant through poor vigor. Furthermore, hybridization techniques are not permitted in traditional wine-growing areas where the production of great wines is based upon a limited number of varieties. Thus, given the strict obligation to ensure trueness of type, *in vitro* methods using vegetative multiplication and somatic embryogenesis have been proposed for grape improvement (Bessis, 1986; Bouquet, 1989). There is, at the present time, much interest in somaclonal variation because it could provide a good means of increasing clonal variation within the traditional cultivars used in Viticulture (Mullins et al., 1992).

Grapevines of most major cultivars have now been regenerated *in vitro* through somatic embryogenesis using nucellar tissues of unfertilised ovules (Mullins & Srinivasan, 1976; Bessis & Labroche, 1985) or the vegetative tissues of anther (Rajasekaran & Mullins, 1983). Regenerated plants arise from diploid cells and should therefore be very similar to the parental plant, while phenotypic and genotypic variants are frequently observed (Grenan, 1982; Mullins, 1985; Bessis, 1986). The variation that arises in tissue cultures may markedly increase the probability of selecting disease resistant varieties (Mullins, 1985; Bessis, 1986; Bouquet, 1989). However, its successful exploitation is dependant upon the availability of reliable procedures. Such tests are already available for *in vitro* selection for resistance to downy mildew (*Plasmopara viticola*) (Lee &

Wicks, 1982; Barlass et al., 1986; Day et al., 1993), dying arm disease (*Eutypa lata*) (Soulié et al., 1993) and powdery mildew (*Uncinula necator*) (Klempka et al., 1984). In contrast, no method is presently available to adequately identify plants derived by somatic embryogenesis with increased levels of resistance to *Botrytis cinerea*.

The objective of the present investigation was thus to establish the basis for a reliable test using the assessment of the phytoalexin precursor resveratrol as a selection criterion to aid the screening of *in vitro*-grown plantlets for grey mould resistance, since we have shown in a preliminary study (Jeandet et al., 1992) that selection at the level of phytoalexin production represents an interesting possibility. Likewise, it is necessary to work with plants cultivated *in vitro*, i.e., in fully controlled conditions bearing in mind that the production of resveratrol by field-grown grapevines has proved to be very sensitive to a wide range of environmental factors (Barlass et al., 1987), which limits its effectiveness as a method of eliminating susceptible progeny. Accordingly, this study aims to achieve two goals: 1) to establish standardized conditions (age of leaves and age of plants) suitable for assaying resveratrol production by grapevine *in vitro* cultures and 2) to study the relationship between resveratrol production by *in vitro* plantlets from 13 *Vitis* spp. or cvs and their field susceptibility to grey mould.

Materials and methods

Plant material and plant cultures

Thirteen grape species or cultivars showing differences in susceptibility to grey mould (data obtained from Galet, 1977) were used in this study:

- Susceptible cultivars of *Vitis vinifera* L.: Pinot noir clone 113, Chardonnay clone 95, Grenache, Carignan, Sémillon clones R1, R4 and R6 (the last three varieties being obtained from INRA Bordeaux).
- Cultivars of *V. vinifera* with intermediate resistance to *B. cinerea*: Cabernet Sauvignon, Sultanine, Sémillon clone R14 (obtained from INRA Bordeaux)
- North American species of *Vitis* tolerant to the disease: *V. labrusca, V. rupestris, V. riparia* cv Gloire de Montpellier.

Plants were cultivated as follows: single-node microcuttings of the 13 *Vitis* spp. were placed in 250 × 20 mm tubes containing 15 ml of a modified Murashige & Skoog medium (1962) (see Fournioux & Bessis, 1993). Temperature of the culture room was 25° C ± 1° C. Light regime was a 16–8 h day – night cycle (ca 100 μE m^{-2} s^{-1}) provided by Sylvania Grolux fluorescent lamps.

Induction of resveratrol synthesis

For this study, UV-irradiation was used as an eliciting agent (Langcake & Pryce, 1977; Jeandet et al., 1991). In order to avoid undesirable effects of UV-irradiation of leaves produced *in vitro* (e.g. toxicity), experiments were performed in which both energy fluence rate and time of exposure to UV-light were reduced when compared with the methods previously described for plants growing in the fields (Langcake & Pryce, 1977; Barlass et al., 1987). In this case, a fluence rate of 400 μW cm^{-2} with exposure of 7 min (0.17 J cm^{-2}) was sufficient to induce stilbene synthesis (Jeandet et al., 1992).

Identification and quantification of resveratrol

Following induction, detached vine leaves were examined under long wave UV-light. *Trans*-resveratrol (hereafter referred as resveratrol) and the biosynthetically related oligomers viniferins are very easily detected at 366 nm since they give characteristic bright blue fluorescence (Langcake & Pryce, 1977). This method thus provides a rapid qualitative assessment of the leaf response to induction.

Quantification of resveratrol was by gas-liquid-chromatography (Jeandet et al., 1991, 1995a) or by high performance liquid chromatography (Jeandet et al., 1995b). Extracts were prepared according to Jeandet et al. (1991).

Results

Standardization of the method: effect of leaf shoot position (age of leaves) and age of in vitro*-grown plantlets on resveratrol production*

In the vine, the aptitude for stilbene production in response to a stress depends on the age of leaves (Langcake & McCarthy, 1979; Langcake, 1981; Pool et al., 1981; Stein & Blaich, 1985): e.g. for plants in the fields, it is maximal in mature (just fully expanded) leaves though it is weak or nil in older and younger leaves. These differences have been correlated to the

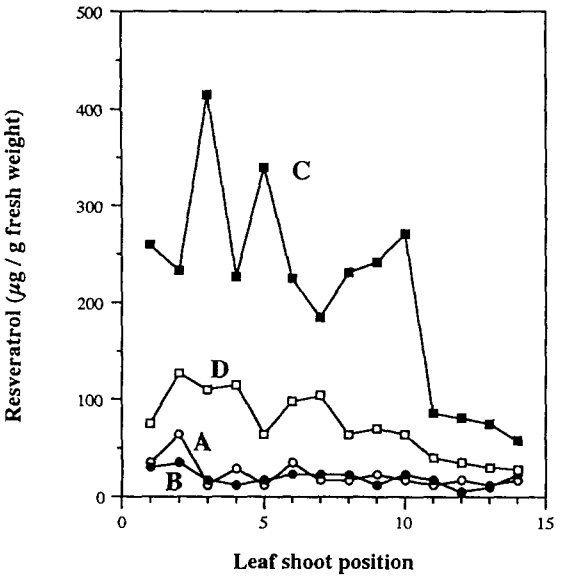

Fig. 1. Effect of leaf shoot position (leaf age) and age of *in vitro*-grown plantlets (cv Cabernet Sauvignon) on resveratrol production. The values represent average resveratrol production of five leaves taken at the same leaf position on the first 14 nodes of different plants grown in the same conditions (leaf shoot position numbered from base). All experiments were done in triplicate. We have given here the results of one significant experiment. Similar results were obtained for the other spp. or cvs. A: 2 month-old-cultures; B: 3 month-old-cultures; C: 4 month-old-cultures; D: 5 month-old-cultures.

Table 1. Resveratrol production in 13 *Vitis* spp. as induced by U.V. irradiation[a]

Vitis spp. or cvs		Resveratrol $\mu g\ g^{-1}$ fresh weight[b] ± SE
cv Chardonnay	G3	122 ± 28
cv Sémillon R1		190 ± 49
cv Pinot noir		224 ± 17
cv Sémillon R4		268 ± 48
cv Sémillon R6		210 ± 20
cv Grenache	G2	257 ± 30
cv Sultanine		277 ± 35
cv Sémillon R14		275 ± 17
cv Cabernet Sauvignon		293 ± 29
V. rupestris	G1	492 ± 28
V. riparia		368 ± 18
V. labrusca		246 ± 49
cv Carignan		382 ± 41

[a] All leaves (except those of the uppermost portion of the plant) of an *in vitro* plantlet (3 plants/experiment) were placed in Petri dishes on moist, filter paper and then irradiated on their abaxial surfaces for 7 min with UV radiation (0.17 J. cm^{-2}). After 24 hours in darkness, resveratrol was extracted according to the method previously described (Jeandet et al., 1991).

[b] Values correspond to the maximal quantities of resveratrol obtained for each species of cultivar tested in response to UV-irradiation. All experiments were repeated 5 times. SE = Standard Errors for the five experiments.

resistance level of grapevine leaves to infection by *Botrytis cinerea* (Langcake & McCarthy, 1979). In the same way, we have sought a developmental stage of *in vitro* plantlets for which inducible quantities of resveratrol reach a maximum. In this aim, the effect of leaf shoot position (age of leaves) and age of the plant on resveratrol synthesis by grapevines was studied.

Two, three, four or five month-old micropropagated plants were respectively used for this study. Every month, resveratrol production of leaves corresponding to every row of insertion on shoot was assessed (Fig. 1). Curves show that quantities of resveratrol inducible from grape leaves reach a maximum for 4-month-old micropropagated plants. In contrast, the capacity to synthesize resveratrol was weak in leaves taken from young or old plantlets (i.e. respectively after 2, 3 or 5 months culture). With regard to the effect of leaf shoot position, one can remark that mature (fully expanded) leaves have higher resveratrol contents (see Fig. 1, curves C and D) than do the young leaves of the uppermost portion of the shoot (from N^{11} to N^{14}). Thus, it seems that for a given time of culture all leaves except the youngest ones, have a relatively high capacity to stilbene production. These observations have led us to express resveratrol synthesis as a function of the age of *in vitro* plantlets without any reference to leaf shoot position.

Production of resveratrol in leaves of different grape species or cultivars grown in vitro

Every month, plantlets of the 13 *Vitis* spp. or cvs were tested for their ability to synthesize resveratrol. Table 1 presents the maximal resveratrol values found for each species or cultivar tested in response to UV-irradiation. This maximum was generally reached for all material in 4 month-old-cultures (see above).

Three groups of plants can be described with respect to the correlation between the potential to produce phytoalexin (i.e. corresponding to the maximal quantities of resveratrol obtained for each species or cultivar tested in response to UV-irradiation) (see Table 1) and resistance:

Group 1: high resveratrol production (from 368 to

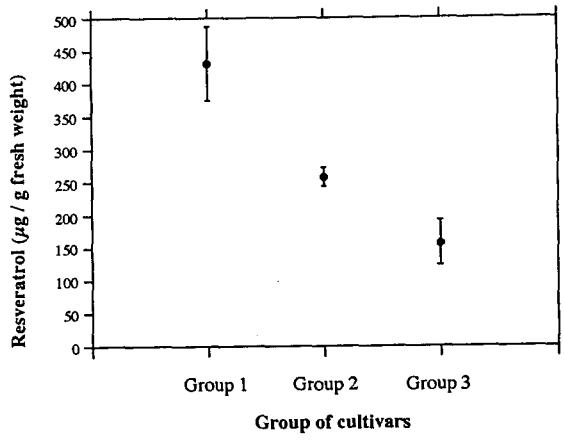

Fig. 2. Resveratrol production measured for the different groups of spp. or cvs: Mean values and 95% confidence intervals. Group 1: *V. riparia* and *V. rupestris* [n = 10]; Group 2: cvs Cabernet Sauvignon, Sultanine, Pinot noir, Grenache and Sémillon (clones R4, R6 and R14) [n = 35]; Group 3: cvs Chardonnay and Sémillon clone R1 [n =10].

492 $\mu g g^{-1}$ fresh weight) + disease tolerance: *Vitis riparia* cv Gloire de Montpellier, *V. rupestris*.

Group 2: intermediate resveratrol production (from 224 to 277 $\mu g g^{-1}$ fresh weight) + low to moderate resistance: cvs Cabernet Sauvignon, Sultanine, Grenache, Pinot noir, Sémillon clones R4, R6 and R14.

Group 3: low resveratrol production (from 122 to 209 $\mu g g^{-1}$ fresh weight) + susceptibility: cvs Chardonnay and Sémillon clone R1.

Figure 2 shows mean values and confidence intervals of resveratrol production measured for the three groups of species or cultivars. Compared with an ANOVA, these values are significantly different ($F = 90.84$, $p = 0.0001$). A complementary analysis by using the Scheffé F-test (a pairwise comparison test adapted for multiple comparisons) indicates that each group of cultivars is significantly different to each other (see Table 2), thus confirming our previous grouping choice (see above).

Differences can thus be observed in the resveratrol production potential among 13 *Vitis* spp. tested in response to UV-irradiation. However, the distinction between intermediate (cvs Cabernet Sauvignon, Sultanine and Sémillon clone R14) and susceptible plants (cvs Grenache, Pinot noir, Sémillon clones R1, R4, R6) was not easily accomplished (Group 2). Our results also showed two plants for which one finds 1) a moderate resveratrol production (ca 246 µg/g fresh weight) along with an elevated disease resistance (*V. labrusca*) and 2) a high capacity for resveratrol synthesis (ca 382 µg/g fresh weight) associated with an elevated susceptibility to grey mould (cv Carignan). When considering these two *Vitis* spp., regression analysis showed (Fig. 3A) a low correlation between resveratrol production by grapevine *in vitro* cultures and grey mould resistance ($y = 172.785 + 41.145 x$ and $R = 0.459$).

Table 2. Values of the Scheffé F-test for the three different pairwise comparisons

Comparison	Scheffé F-test
Group 1 versus Group 2	53.67*
Group 1 versus Group 3	87.15*
Group 2 versus Group 3	18.64*

* : values significantly different at the 0.05 level.

The existence of plants tolerant to the disease despite an inability to synthesize high phytoalexin concentrations suggests that resistance of grapevines to grey mould may sometimes be associated with factors other than stilbene phytoalexins. Such a situation has already been reported by Stein & Blaich (1985) and Dercks & Creasy (1989). On the other hand, an elevated susceptibility to grey mould associated with a high capacity for stilbene synthesis (cv Carignan) can be explained by the fact that this cultivar has very tightly-packed bunches (Galet, 1977). This infers, on the one hand, the presence of confined and humid regions inside grape clusters which favour aggressiveness of the fungus and, on the other hand, formation by squashing of polyhedric berries, which in certain cases can separate from their pedicels, thus forming preferential sites for *Botrytis* attacks. If one excludes these two plants, regression analysis based on 11 species or varieties shows a good correlation between resveratrol synthesis and grey mould resistance ($y = 51.292 + 86.135 x$ $R = 0.831$, see Fig. 3B).

Phytoalexin response is thus one of the important factors implied in the resistance of grapevines to grey mould. Obviously, many other factors can probably interplay in determining the outcome of the *Botrytis cinerea*-grapevine interaction. Nevertheless, the good correlation found among 11 *Vitis* spp. or cvs allows us to validate the choice we have made in studying this system.

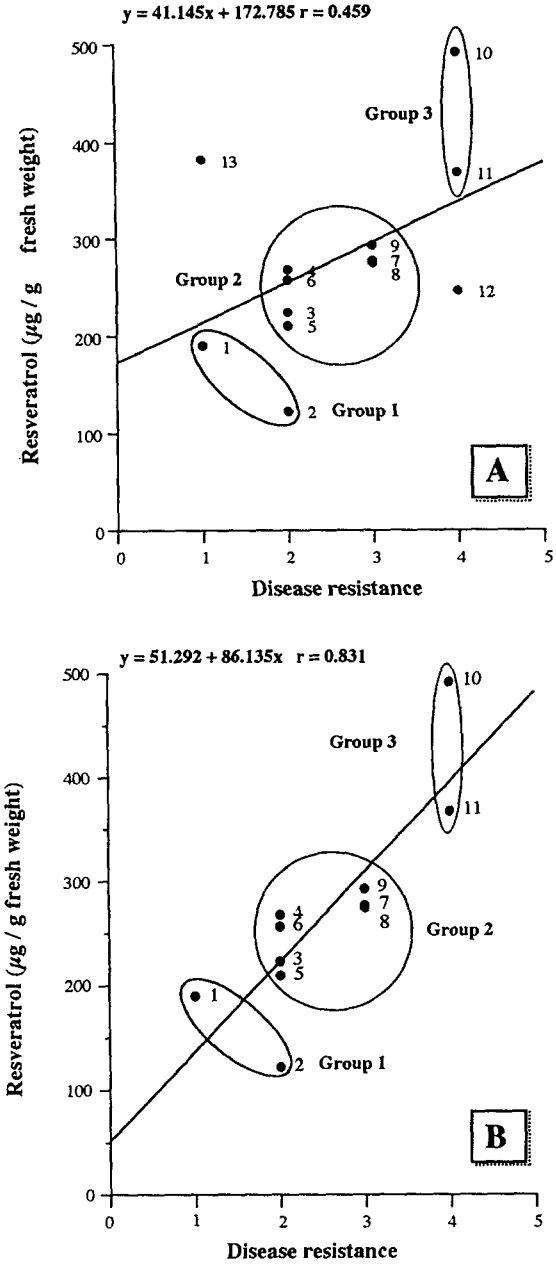

Fig. 3. Relationship between resveratrol production by *in vitro* plantlets and their field susceptibility to *Botrytis cinerea*: (A) Regression analysis based on 13 *Vitis* spp. or cvs. (B) Regression analysis based on 11 *Vitis* spp. or cvs. 1: cv Sémillon clone R1; 2: cv Chardonnay; 3: cv Pinot noir; 4: cv Grenache; 5: cv Sémillon clone R6; 6: cv Sémillon clone R4; 7: cv Sémillon clone R14; 8: cv Cabernet Sauvignon; 9: cv Sultanine; 10: *V. rupestris*; 11: *V. riparia*; 12: *V. labrusca*; 13: cv Carignan. Disease resistance was rated as follows (data obtained from Galet, 1977): 1 = very low resistance; 2 = low resistance; 3 = intermediate resistance; 4 = high resistance.

Discussion

This study has shown that a short UV-irradiance of *in vitro* plantlets can stimulate phytoalexin (resveratrol) synthesis. To our knowledge, this finding is unprecedented in that UV-irradiation has previously been reported to be unsuitable for the induction of *in vitro* plantlets (see Barlass et al., 1987).

In vitro stimulation of defence responses and namely of stilbene phytoalexin production has already been described in cultured grapevine cells using fragments of fungal cell wall as elicitors (Hoos & Blaich, 1988; Liswidowati et al., 1991; Calderon et al., 1993). Nevertheless, the fact that vine regeneration from a single cell has not yet been achieved (Skene, 1975; Hoos & Blaich, 1988; Barbier & Bessis, 1990), limits the usefulness of the elicitation of cell suspensions as a possible test for the breeding of resistant varieties. In contrast, we described here a method permitting direct and quantitative estimation of resveratrol production in leaves of *in vitro* plantlets. A good correlation (R = 0.831) exists between the potential to produce resveratrol in response to UV-irradiation and resistance among 11 *Vitis* spp. or cvs. These results should however be interpreted cautiously since no correlation was found for *V. labrusca* and cv Carignan. This is not surprising insofar as resistance of grapevines to *B. cinerea* is probably of polygenic nature. This could explain why the resistance of *V. labrusca* is coupled with a moderate ability to produce resveratrol. Finally, the fact that susceptibility is associated with high phytoalexin production (cv Carignan) indicates that varietal factors such as grape cluster architecture could also take place in the resistance of grapevines to *B. cinerea* (Vail & Marois, 1991). If one excludes these two plants, it therefore appears that resveratrol can be considered as a good marker for grey mould resistance and would be able to serve as a means to screen for the classifying of susceptible and resistant varieties in a strategy of selection.

Finally, phytoalexins have long been referred as being important in the defence of plants against fungal infection. In this study, we have shown that in particular the phytoalexin resveratrol plays a role in the resistance to grape diseases. The biosynthesis of this phytoalexin is controlled by stilbene synthase (Schöppner & Kindl, 1984). Recently, Hain et al. (1990 and 1993) have isolated stilbene synthase genes from grapevines and transferred them into tobacco plants where their expression resulted in synthesis of resveratrol. Stilbene synthase gene transfer experiments into grapevines are

now being studied (M. Boulay & H. Kindl, personnal communication) in order to increase disease resistance of grapevines to grey mould.

Through this study, we have developed a method for evaluating the physiological expression of stilbene synthase gene in grapevines under fully controlled conditions, which would aid the analysis of stilbene synthase gene expression (over-expression?) in transgenic plants.

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